ISSN 1346-7565 Acta Phytotax. Geobot. 66 (1): 1–9 (2015)

Genetic Diversity and Structure of *Euryale ferox* Salisb. (Nymphaeaceae) in Japan

Ayumi Imanishi[1], Shingo Kaneko[2], Yuji Isagi[3], Junichi Imanishi[4], Yosihiro Natuhara[5] and Yukihiro Morimoto[6]

*[1] Faculty of Applied Sociology, Kindai University, 228-3 Shinkamikosaka, Higashiosaka, Osaka 577-0813, Japan. *ayumiimanishi@gmail.com (author for correspondence); [2] Faculty of Symbiotic Systems Science and Technology, Fukushima University, Kanayagawa 1, Fukushima, Fukushima 960-1248, Japan; [3] Graduate School of Agriculture, Kyoto University, Oiwake-cho, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan; [4] Graduate School of Global Environmental Studies, Kyoto University, Yoshida-Honmachi, Sakyo-ku, Kyoto 606-8501, Japan; [5] Graduate School of Environmental Studies, Nagoya University, Furo-cho, Chikusa-ku, Nagoya, Aichi 464-8601, Japan; [6] Faculty of Bio-environmental Science, Kyoto Gakuen University, 1-1 Nanjo-Otani, Sogabe, Kameoka, Kyoto 621-8555, Japan*

We analyzed the population genetic structure of *Euryale ferox* Salisb. (Nymphaeaceae), an annual aquatic herb, to obtain information useful for conservation of this species. A total of 391 plants from 58 populations throughout Japan and four plants from a population in China were sampled and genotyped using eight microsatellite markers. Within- and among-population genetic diversity of *E. ferox* in Japan was basically low, with all eight microsatellite loci fixed to single alleles in many populations. However, multilocus genotypes including heterozygous loci were detected from three populations; these populations had inbreeding coefficients (F_{IS}) not equal to 1.0 and it is assumed that these multilocus genotypes are produced by outbreeding of genetically different individuals. Principal coordinate analysis (PCO) and population genetic structure analysis using INSTRUCT software uncovered at least two genetically distinct groups within Japanese *E. ferox* populations, neither of which had a simple geographical distribution pattern. This complex genetic structure may be the result of a random fixation of MLGs through genetic drift after habitat loss and by spatial and temporal admixture among populations through seed dispersal and seed banks.

Keywords: aquatic plant, conservation, gene flow, genetic drift, microsatellite marker, outbreeding, seed bank, seed dispersal, self-fertilization, vulnerable species

Aquatic plant populations in Japan are decreasing as a result of human activities such as land reclamation and water pollution (Kadono & Yuma 1995). Approximately 40% of all aquatic plants in Japan (about 90 species) are listed as endangered or vulnerable in the Red List of Threatened Plants of Japan (Tanaka 2012).

Euryale ferox Salisb. (Nymphaeaceae) is an annual aquatic herb with gigantic prickly floating leaves. It is distributed from northwestern India to Japan (Kadono 1994a) and inhabits meso- and eutrophic water bodies such as lakes, ponds, reservoirs, and rivers. In Japan, the species' distribution ranges from the northern part of Honshu to Kyushu, with about 300 populations previously recorded (Kadono 1994b). The number of populations has recently declined to around 80 because of habitat loss and degradation (Fukushimagata

Lagoon Water Park 2007), and the species is classified as "vulnerable" in the Red List of Threatened Plants of Japan (Ministry of the Environment of Japan 2012). *Euryale ferox* populations have also declined throughout its global range (Schneider *et al*. 2003).

Genetic diversity loss may reduce the ability of populations to evolve in response to environmental change and increase their risk of extinction. Genetic information is therefore important for endangered species conservation planning and restoration strategies (Ellstrand & Elam 1993, Newman & Pilson 1997, Frankham & Ralls 1998). As an example, restoration efforts involving translocation of endangered plant species from non-local populations must consider potential risks such as genetic swamping and outbreeding depression due to introduction of foreign genotypes (Hufford & Mazer 2003).

Previous ecological research has revealed that *Euryale ferox* reproduces mainly by selfing (Okada & Otaya 1930, Kadono & Schneider 1987). This result suggests that *E. ferox* possesses a high level of population differentiation, requiring elucidation of the genetic condition of each population or individual for effective species conservation. Koren *et al*. (2012) studied *E. ferox* in the southern Russian Far East, and observed a low level of allozyme variation. The genetic diversity of this species has not been examined, however, with highly variable and informative genetic markers, such as microsatellite markers widely applied in other species (Ballou & Lugon-Moulin 2002). To obtain more accurate and useful information for the conservation of *E. ferox*, in the present study we used microsatellite markers to analyze its population genetic structure.

Materials and Methods

Plant sampling

In July, August, and September of 2008 and 2009, we collected leaves of *Euryale ferox* from 58 populations covering its entire Japanese distributional range and a population in the Jingxin Wetland of northeastern China (Table 1). Populations in separated ponds or canals were considered to be different. A total of 395 randomly sampled individuals were collected. Plant materials were kept on ice after field collection and then frozen at −30°C in the laboratory until DNA extraction.

Microsatellite genotyping

Genomic DNAs of all samples were extracted from leaves using a modified CTAB method (Milligan 1992). Eight microsatellite loci (*Efer004, Efer013, Efer015, Efer023, Efer032, Efer045, Efer047*, and *Efer136*) developed for *Euryale ferox* (Imanishi *et al*. 2011) were amplified by PCR using a PCR Qiagen Multiplex PCR kit (Qiagen, Valencia, CA, USA) in 10-μL reaction volumes containing 5 ng extracted DNA, 5 μL of 2× Multiplex PCR Master Mix, and 0.2 μM of each multiplexed primer. PCR amplifications were performed in a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems, Carlsbad, CA, USA) using the following conditions: initial denaturation at 95°C for 15 min, followed by 25 or 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 1 min 30 s, and extension at 72°C for 1 min, and a final extension at 60°C for 30 min. PCR product sizes were measured using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) and Genotyper analysis software (Applied Biosystems).

Data analysis

We sampled and genotyped 395 plants from 59 populations. To estimate genetic diversity within each population, mean number of alleles per locus (N_A) and observed and expected heterozygosities (H_O and H_E) were calculated using GenAlEx ver. 6.5 (Peakall & Smouse 2012). In addition, allelic richness (A_R; El Mousadik & Petit 1996) and inbreeding coefficient (F_{IS}) were calculated using FSTAT ver. 2.9.3 (Goudet 2001). Multilocus genotypes (MLGs) were defined based on the combination of genotypes of the eight loci. Using GenAlEx ver. 6.5 (Peakall & Smouse 2012), principal coordinate analysis (PCO) was performed to evaluate genetic relationships among MLGs based on two genetic distances: D_{ps}, which is based on the number of shared al-

TABLE 1. Sampling locations, number of individuals genotyped, genetic diversity, and MLGs identified within populations of *Euryale ferox*.

No.	Population	Location	Lat. (N)	Long. (E)	Water area (ha)	n	N_A	A_R	H_O	H_E	F_{IS}	N_G	MLGs
Hokushinetsu													
1	Niigata A	Niigata City, Niigata	37.91	139.25	193.0	8	1.9	1.4	0.156	0.342	0.59	6	A, B, C, D, E, F
2	Niigata B	Niigata City, Niigata	37.82	138.88	31.0	8	1.0	1.0	0.000	0.000	---	1	G
3	Joetsu	Joetsu City, Niigata	37.14	138.35	2.5	8	1.1	1.0	0.000	0.027	1.00	2	G, H
4	Himi	Himi City, Toyama	36.84	136.97	5.5	8	1.0	1.0	0.000	0.000	---	1	F
Kanto													
5	Tsuchiura	Tsuchiura City, Ibaraki	36.08	140.16	2.4	8	1.0	1.0	0.000	0.000	---	1	R
6	Kazo	Kazo City, Saitama	36.18	139.66	0.6	8	1.0	1.0	0.000	0.000	---	1	G
7	Tatebayashi A	Tatebayashi City, Gunma	36.23	139.54	0.2	8	1.9	1.2	0.109	0.196	0.50	3	F, I, J
8	Tatebayashi B	Tatebayashi City, Gunma	36.22	139.53	0.2	8	1.0	1.0	0.000	0.000	---	1	G
9	Katsushika	Katsushika-ku, Tokyo	35.79	139.87	0.1	8	1.1	1.0	0.000	0.027	1.00	2	F, K
Tokai													
10	Shizuoka	Shizuoka City, Shizuoka	35.02	138.39	6.8	8	1.0	1.0	0.000	0.000	---	1	L
11	Fujieda	Fujieda City, Shizuoka	34.88	138.26	4.5	8	1.0	1.0	0.000	0.000	---	1	L
12	Kakegawa A	Kakegawa City, Shizuoka	34.69	138.06	7.1	2	1.0	1.0	0.000	0.000	---	1	L
13	Kakegawa B	Kakegawa City, Shizuoka	34.67	138.02	0.6	8	1.0	1.0	0.000	0.000	---	1	L
14	Kaizu	Kaizu City, Gifu	35.18	136.66	5.5	8	1.0	1.0	0.000	0.000	---	1	L
15	Higashiura	Chita County, Aichi	34.98	136.96	0.1	8	1.1	1.1	0.000	0.059	1.00	2	L, M
Kinki													
16	Hikone	Hikone City, Shiga	35.28	136.25	0.9	8	1.0	1.0	0.000	0.000	---	1	M
17	Kameoka	Kameoka City, Kyoto	35.07	135.57	8.7	8	1.0	1.0	0.000	0.000	---	1	M
18	Kyoto	Kyoto City, Kyoto	34.92	135.74	0.1	5	1.0	1.0	0.000	0.000	---	1	M
19	Ibaraki	Ibaraki City, Osaka	34.81	135.55	0.6	1	1.0	1.0	0.000	0.000	---	1	M
20	Kobe	Kobe City, Hyogo	34.74	134.98	1.6	1	1.0	1.0	0.000	0.000	---	1	M
21	Akashi A	Akashi City, Hyogo	34.69	134.91	6.5	8	1.9	1.4	0.000	0.414	1.00	3	M, N, Q
22	Akashi B	Akashi City, Hyogo	34.68	134.91	2.1	8	1.1	1.0	0.000	0.027	1.00	2	Q, O
23	Akashi C	Akashi City, Hyogo	34.69	134.92	1.7	1	1.0	1.0	0.000	0.000	---	1	P
24	Akashi D	Akashi City, Hyogo	34.72	134.90	0.8	8	1.0	1.0	0.000	0.000	---	1	R
25	Akashi E	Akashi City, Hyogo	34.69	134.92	0.9	1	1.0	1.0	0.000	0.000	---	1	M
26	Akashi F	Akashi City, Hyogo	34.67	134.94	1.1	8	1.0	1.0	0.000	0.000	---	1	Q
Chugoku													
27	Bizen	Bizen City, Okayama	34.74	134.14	18.5	2	1.0	1.0	0.000	0.000	---	1	R
28	Okayama A	Okayama City, Okayama	34.57	133.93	100.0	8	1.0	1.0	0.000	0.000	---	1	M
29	Okayama B	Okayama City, Okayama	34.70	133.87	1.8	8	1.0	1.0	0.000	0.000	---	1	M
30	Okayama C	Okayama City, Okayama	34.70	134.03	1.2	1	1.0	1.0	0.000	0.000	---	1	R
31	Okayama D	Okayama City, Okayama	34.71	134.07	1.6	8	1.0	1.0	0.000	0.000	---	1	M
32	Kurashiki A	Kurashiki City, Okayama	34.53	133.64	4.2	1	1.0	1.0	0.000	0.000	---	1	M
33	Kurashiki B	Kurashiki City, Okayama	34.65	133.70	4.7	8	1.0	1.0	0.000	0.000	---	1	M
34	Asaguchi	Asaguchi City, Okayama	34.55	133.63	3.9	8	1.0	1.0	0.000	0.000	---	1	M
35	Fukuyama A	Fukuyama City, Hiroshima	34.52	133.39	3.8	8	1.0	1.0	0.000	0.000	---	1	M
36	Fukuyama B	Fukuyama City, Hiroshima	34.56	133.36	3.8	8	1.0	1.0	0.000	0.000	---	1	M
Shikoku													
37	Sakaide	Sakaide City, Kagawa	34.29	133.84	5.2	8	1.0	1.0	0.000	0.000	---	1	R
38	Marugame A	Marugame City, Kagawa	34.25	133.81	1.8	8	1.0	1.0	0.000	0.000	---	1	R
39	Marugame B	Marugame City, Kagawa	34.25	133.83	6.6	8	1.0	1.0	0.000	0.000	---	1	R
40	Zentsuji A	Zentsuji City, Kagawa	34.23	133.78	1.4	8	1.0	1.0	0.000	0.000	---	1	R
41	Zentsuji B	Zentsuji City, Kagawa	34.24	133.78	0.7	8	1.0	1.0	0.000	0.000	---	1	R
42	Tadotsu	Tadotsu City, Kagawa	34.26	133.76	3.1	8	1.0	1.0	0.000	0.000	---	1	R
43	Kanonji	Kanonji City, Kagawa	34.10	133.71	0.7	8	1.0	1.0	0.000	0.000	---	1	R
44	Naruto A	Naruto City, Tokushima	34.16	134.55	1.3	8	1.0	1.0	0.000	0.000	---	1	M
45	Naruto B	Naruto City, Tokushima	34.16	134.51	0.1	8	1.0	1.0	0.000	0.000	---	1	M
46	Naruto C	Naruto City, Tokushima	34.15	134.59	0.1	8	1.0	1.0	0.000	0.000	---	1	M
47	Naruto D	Naruto City, Tokushima	34.16	134.59	0.1	3	1.0	1.0	0.000	0.000	---	1	M
48	Naruto E	Naruto City, Tokushima	34.16	134.59	0.1	8	1.0	1.0	0.000	0.000	---	1	M
49	Naruto F	Naruto City, Tokushima	34.16	134.60	0.1	8	1.0	1.0	0.000	0.000	---	1	M
Kyushu													
50	Usa	Usa City, Oita	33.55	131.26	1.8	8	1.0	1.0	0.000	0.000	---	1	R
51	Nakatsu	Nakatsu City, Oita	33.55	131.26	1.1	8	1.0	1.0	0.000	0.000	---	1	R
52	Yukuhashi	Yukuhashi City, Fukuoka	33.69	130.97	6.5	8	1.4	1.2	0.219	0.150	-0.40	2	S, T
53	Yamaga	Yamaga City, Kumamoto	33.04	130.66	0.3	8	1.0	1.0	0.000	0.000	---	1	R
54	Saga	Saga City, Saga	33.24	130.30	2.1	8	1.0	1.0	0.000	0.000	---	1	R
55	Satsumasendai A	Satsumasendai City, Kagoshima	31.82	130.19	0.8	1	1.0	1.0	0.000	0.000	---	1	R
56	Satsumasendai B	Satsumasendai City, Kagoshima	31.84	130.25	0.6	8	1.0	1.0	0.000	0.000	---	1	R
57	Minamitane A	Minamitane-cho, Kagoshima	30.44	130.95	0.4	8	1.0	1.0	0.000	0.000	---	1	R
58	Minamitane B	Minamitane-cho, Kagoshima	30.38	130.96	0.2	4	1.0	1.0	0.000	0.000	---	1	R
China													
59	Jingxin	Hunchun City, Jilin Province, China	41.62	130.57	-	4	1.1	1.1	0.125	0.063	-1.00	1	U
Mean							1.1	1.0	0.010	0.022	0.52	1.2	

n, number of individuals genotyped; N_A, mean number of alleles per locus; A_R, allelic richness; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; N_G, number of MLGs

TABLE 2. Twenty-one MLGs based on the combination of genotypes of the eight loci of *Euryale ferox* and number of populations including each MLG.

MLGs	Number of populations including each MLG	Genotypes at each locus*															
		Efer004		Efer013		Efer015		Efer023		Efer032		Efer045		Efer047		Efer136	
A	1	162	162	125	127	163	165	168	168	158	160	222	222	108	108	218	218
B	1	162	162	125	125	163	165	170	170	158	160	224	224	94	108	214	214
C	1	162	162	125	127	165	165	168	168	158	160	224	224	94	108	218	218
D	1	162	162	127	127	165	165	170	170	158	158	222	224	94	94	214	214
E	1	162	162	127	127	165	165	168	168	158	158	222	222	108	108	214	214
F	4	162	162	127	127	165	165	168	168	160	160	222	222	108	108	218	218
G	4	162	162	125	125	163	163	170	170	158	158	224	224	94	94	214	214
H	1	162	162	127	127	163	163	170	170	158	158	224	224	94	94	214	214
I	1	162	162	125	127	163	165	168	170	158	158	222	224	94	94	214	214
J	1	162	162	125	127	165	165	168	170	158	160	222	222	108	108	214	214
K	1	162	162	127	127	165	165	168	168	160	160	224	224	108	108	218	218
L	5	162	162	123	123	163	163	170	170	158	158	224	224	94	94	214	214
M	22	162	162	123	123	163	163	170	170	160	160	224	224	94	94	214	214
N	1	162	162	123	123	165	165	170	170	160	160	224	224	94	94	214	214
O	1	164	164	127	127	163	163	168	168	160	160	222	222	108	108	218	218
P	1	162	162	123	123	163	163	170	170	160	160	222	222	94	94	214	214
Q	3	164	164	127	127	165	165	168	168	160	160	222	222	108	108	218	218
R	18	162	162	125	125	163	163	170	170	160	160	224	224	94	94	214	214
S	1	162	162	125	125	163	163	168	168	160	160	222	222	108	108	214	214
T	1	162	162	125	125	163	163	170	170	160	160	222	224	94	108	214	214
U	1	160	160	125	125	165	165	168	168	160	160	222	222	108	108	214	218
Number of alleles per locus		3		3		2		2		2		2		2		2	

*, Alleles of heterozygous loci are framed by dashed lines.

leles summed over loci (Bowcock *et al*. 1994), and D_{dm}, which incorporates a stepwise mutation model (Goldstein *et al*. 1995). Pairwise matrices of these genetic distances were constructed by Microsatellite Analyser ver. 4.05 (Dieringer & Schlötterer 2003). We also used Bayesian clustering to evaluate population genetic structure using INSTRUCT (Gao *et al*. 2007), which does not assume Hardy-Weinberg equilibrium. We performed 20 independent runs for each value of *K* from 1 to 8 under a population structure model allowing for admixture. Each run consisted of 1,000,000 iterations after an initial burn-in of 100,000 iterations. The optimum number of clusters was determined by comparing the log likelihood and deviance information criterion in each *K*.

Results

Genetic diversity of the populations in Japan

Genetic analysis using the eight microsatellite loci revealed generally low genetic diversity within *Euryale ferox* populations (Table 1). The number of alleles per locus (N_A) ranged from 1.0 to 1.9, with an average of 1.1. Within-population allelic richness (A_R) ranged from 1.0 to 1.4, with an average of 1.0. Average observed heterozygosity (H_O) and expected heterozygosity (H_E) within each population ranged respectively from 0.00 to 0.22, with an average of 0.01, and from 0.00 to 0.41, with an average of 0.02. The value of the inbreeding coefficient (F_{IS}) was not equal to 1.0 in Niigata A (Pop. 1), Tatebayashi A (Pop. 7), and Yukuhashi (Pop. 52) populations. Based on the eight microsatellite loci, 20 MLGs were detected in the Japanese populations. One MLG observed at the Jingxin Wetland in China (Pop. 59) was not observed in Japanese populations. Fifty of 58 populations in Japan were characterized by single MLGs. More than two MLGs were found in eight populations, and an exceptionally large number (6) were observed in the Niigata A population (Pop. 1). MLGs including heterozygous loci rep-

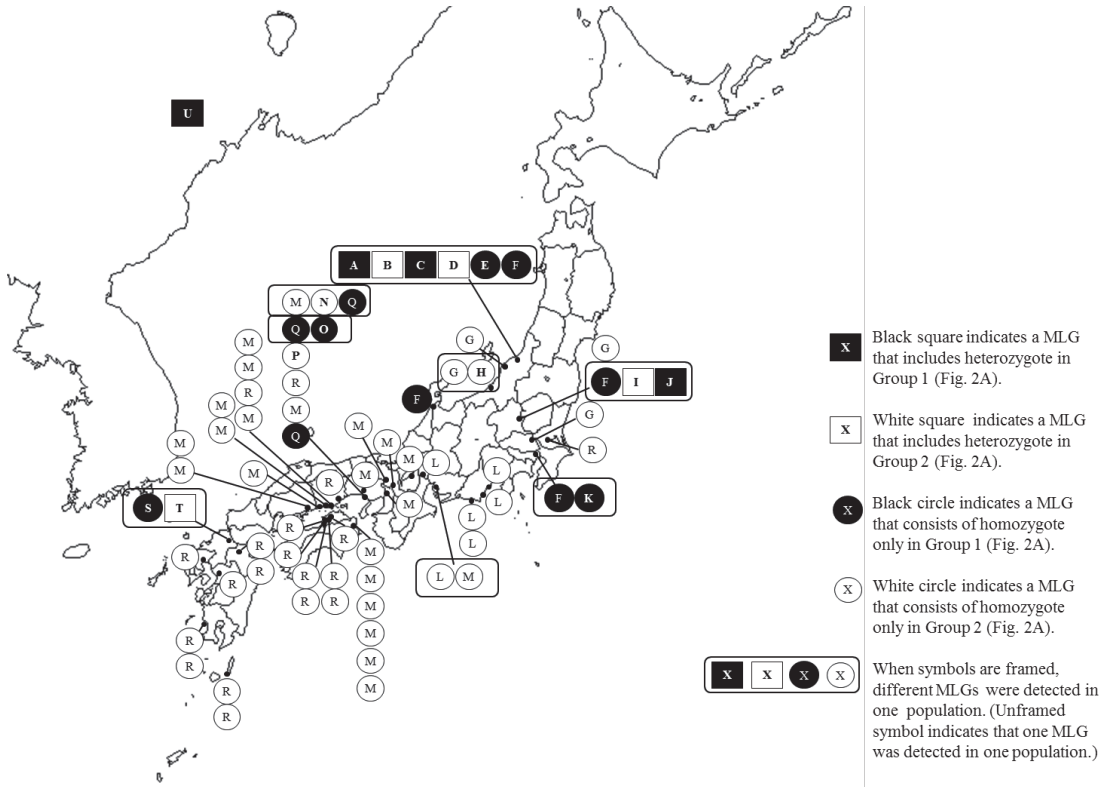

FIG. 1. Geographical distribution of 21 different multilocus genotypes (MLGs) based on combinations of genotypes of eight loci present in 59 populations of *Euryale ferox*. Letters A to U correspond to the 21 different MLGs.

resented eight of 21 MLGs; the remaining 13 were homozygous at all loci examined (Table 2).

Spatial distribution of genotypes and their genetic similarity

Different MLG distribution patterns could be roughly discerned between eastern and western Japanese populations (Fig. 1 and Table 1). In eastern Japan, comprising Hokushinetsu and Kanto regions (Pops. 1–9), two MLGs, F and G, were observed with relatively high frequency (Table 1). In western Japan, consisting of Tokai, Kinki, Chugoku, Shikoku, and Kyushu regions (Pops. 10–58), MLGs M and R were widely distributed and detected in many populations (Table 1). Exceptionally, MLG R was also found in the Tsuchiura population (Pop. 5) in the Kanto region (Table 1). MLG L was observed in all Tokai region populations (Pops. 10–15; Table 1).

In the PCO using D_{ps}, the first PCO axis explained half of the variation (49.3%) and separated the 21 MLGs into two genetic groups, Groups 1 and 2 (Fig. 2A). In the case of D_{dm}, three distinct groups were detected at PCO axis 1, which explained most of the variation (89.2%) (Fig. 2B). Geographical distribution of genetic groups detected by PCO showed no clear spatial structure (Fig. 1).

INSTRUCT analysis indicated that populations of *Euryale ferox* are divided into distinct genetic clusters (Fig. 3). The highest log likelihood and the lowest deviance information criterion were both obtained at $K = 4$ (Fig. 4). Most individuals were separated into distinct clusters, with a small number of admixed individuals in Niigata A (Pop. 1), Joetsu (Pop. 3) Tatebayashi A (Pop.7),

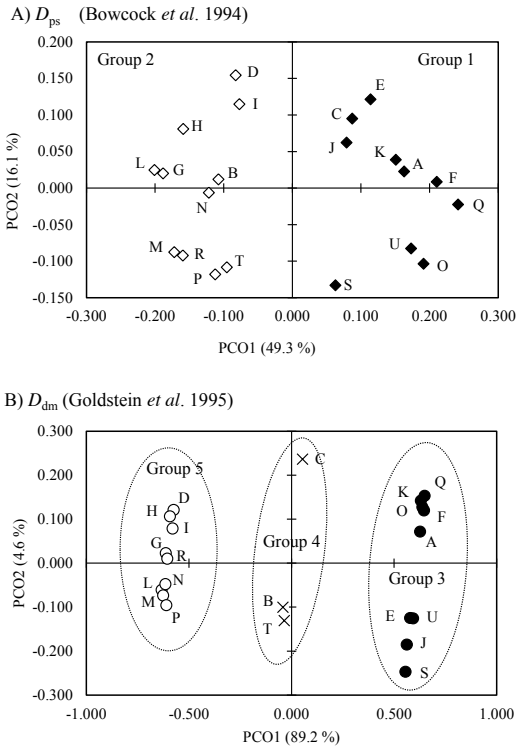

FIG. 2. Plots of first and second principal coordinates obtained from genetic distances (A) D_{ps} (Bowcock *et al*. 1994) and (B) D_{dm} (Goldstein *et al*. 1995) between 21 multilocus genotypes (MLGs) of *Euryale ferox*. Different symbols represent different groups.

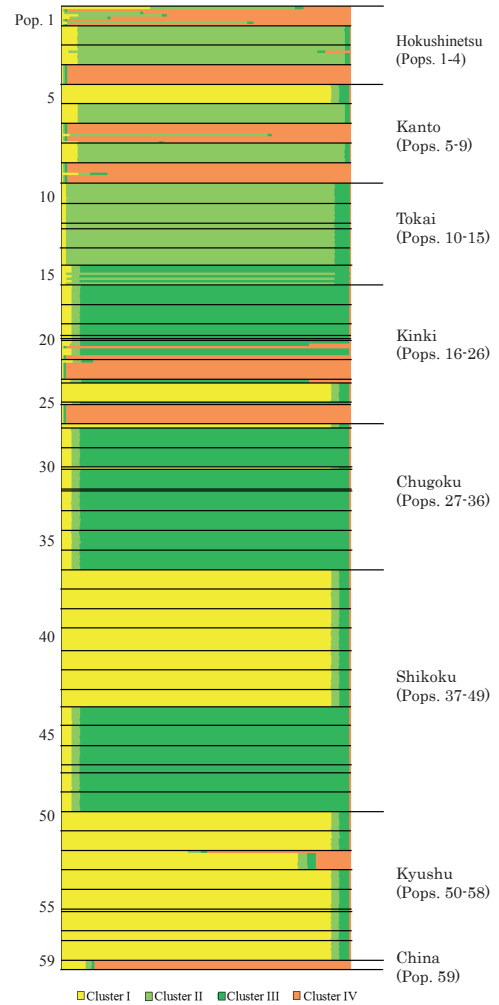

FIG. 3. INSTRUCT analysis with K=4 for 395 individuals from 59 populations of *Euryale ferox*.

Katsushika (Pop. 9), Akashi A (Pop. 21), Akashi C (Pop. 23), and Yukuhashi (Pop. 52) populations (Fig. 3). While the distribution of individuals assigned to clusters I, II, and III was geographically biased, individuals assigned with high probability to cluster IV showed a disjunct distribution and were observed in populations from Hokushinetsu, Kanto, and Kinki regions of Japan and from China (Fig. 3).

Discussion

Genetic diversity and structure of Euryale ferox

Although microsatellites derived from the genomic libraries usually have high levels of polymorphism (Cho *et al*. 2000), only two to three alleles per locus were detected in each sample (Table 2). Among- and within-population genetic diversity of *Euryale ferox* in Japan was basically low, with all eight microsatellite loci fixed to single alleles in many populations (Table 1). These results are consistent with previous genetic studies of *E. ferox*, which showed extremely low levels of allozyme variation (Koren *et al*. 2012) and remarkably low microsatellite heterozygosity (Quan *et al*. 2009). Most flowers of *E. ferox* are cleistogamous (Okada & Otaya 1930), and although chasmogamous flowers are also produced, self-pollination occurs prior to anthesis (Kadono

& Schneider 1987). Possible factors leading to the fixation of alleles within these populations are repeated self-fertilization based on the reproductive characteristics, an annual life history, small population size, and repeated bottlenecks or founder events.

Our results suggest that outbreeding has occurred in Japanese *Euryale ferox* populations. MLGs including heterozygous loci were detected in three populations—Niigata A, Tatebayashi A, and Yukuhashi (Fig. 1), and the F_{IS} values of these populations were not equal to 1.0 (Table 1). MLGs A, B, C, I, and J were heterozygous at three or four of the eight microsatellite loci (Table 2). Because the possibility that these genotypes were maintained by selfing or were created by mutation is low, it is assumed that these MLGs in the three populations are produced by outbreeding of genetically different individuals. In most populations, however, all loci were monomorphic. Thus, any outbreeding that occurred in those populations could not be detected.

Spatial genetic structure is influenced by various historical and ecological factors that affect the amount of genetic drift and gene flow (Hutchison & Templeton 1999). Because the distribution of some MLGs (F, G, L, M, and Q) detected in multiple populations was geographically biased, panmictic gene flow among the regions may be unlikely. PCO and INSTRUCT analysis uncovered at least two genetically distinct groups among Japanese *Euryale ferox* populations (Figs. 2 and 3), neither of which had a simple geographical distribution pattern (Fig. 1). Although a scenario explaining the current distributional pattern of the two genetic groups distinguished by PCO analysis (Fig. 2A) cannot be easily envisioned, complex genetic structure, especially in eastern Japan, may be influenced by a random fixation of MLGs through genetic drift after habitat loss and by spatial and temporal admixture among populations through seed dispersal and seed banks. *E. ferox* populations in Japan seem to be strongly affected by genetic drift caused by sharp declines in population numbers and sizes and the reproductive ecology mentioned above. In addition, although no genetic marker-based

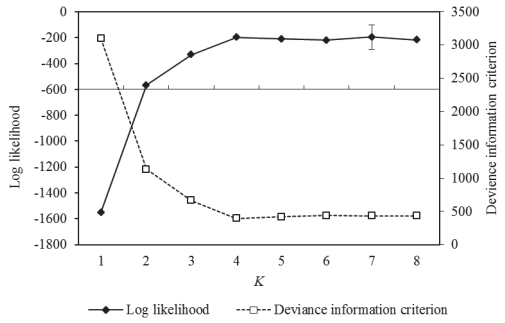

FIG. 4. Estimated log-likelihood and deviance information criterion values over 20 independent INSTRUCT runs (mean ± SD) for K = 1 to 8 population clusters.

study directly addressing gene flow in *Euryale ferox* has been conducted, our discovery of an identical MLG (R) in populations separated by more than 200 km, and the fact that new *E. ferox* populations are occasionally discovered—such as in Tanabe City in Wakayama Prefecture (Goto 2009) and Wajima City in Ishikawa Prefecture (Takagi and Honda 2012), provided indirect but reasonable evidence of possible seed dispersal by water birds. Furthermore, populations of *E. ferox* possibly arising through seed bank have been often rediscovered (Hada 1988, Matsumoto 2002). Seed dispersal and seed banks may thus contribute to spatial and temporal admixture among *E. ferox* populations.

Implications for conservation

The findings of our study provide useful information for the conservation of *Euryale ferox*, especially with respect to its genetic diversity. Nonetheless, the monomorphic MLGs uncovered by our study may have variation at other loci, as only eight loci were examined and genome-wide variation in *E. ferox* is still unknown. Genetic diversity levels differed among populations (Table 1 and Fig. 1). Consequently, the populations showing high genetic diversity and including rare MLGs (Niigata A, Joetsu, Tatebayashi A, Katsushika, Akashi A, Akashi B, and Yukuhashi populations) should be given high priority for effective conservation of remaining *E. ferox* genetic diver-

sity. In regard to populations with multiple MLGs, habitat-degrading activities, such as reclamation and water pollution, should be avoided to prevent the erosion of genetic diversity due to the reduction in population sizes including seed banks. In addition to the conservation of standing genetic variations, our study suggests the possibility of regeneration of genetic variations by means of outbreeding among spatial or temporal migrants, followed by recombination among loci and subsequent selfing. Thus, the maintenance of habitat conditions facilitating this process may be important. Considering the current situation of sharp decline in *E. ferox* population numbers and sizes, *ex situ* conservation may be needed to preserve the MLGs observed with low frequency. Furthermore, given that outbreeding depression is theoretically possible (Parker 1992, Dolgin *et al*. 2007), artificial migration and admixture must be prevented to avoid disruption of locally-adapted homozygous MLGs.

Genetic analysis of recently discovered or rediscovered populations may be an efficient way to detect unknown genotypes: these populations may be derived from seed banks, which can maintain genetic variations that have disappeared from existing populations (e.g., Uesugi *et al*. 2007), and/or from seeds dispersed from other populations. For example, although the rediscovered Kameoka and Kyoto populations had a common MLG (M) in the region (Table. 1), the Niigata A population at Fukushimagata Lagoon, which had rare MLGs and the highest genetic diversity of populations analyzed in this study (Table 1), had experienced extinction once in the 1960s because of reclamation and water pollution (Fukushimagata Lagoon Water Park 2007). However, some individuals were rediscovered in 1988, and then conservation efforts have been carried out by the local government and citizens (Fukushimagata Lagoon Water Park 2007). It has been suggested that the present Niigata A population originated from a seed bank and/or seed dispersal. The genetically variable seed source and large habitat area (193 ha) may help maintain its high genetic diversity.

We are grateful to all the local governments and citizens conserving *Euryale ferox*, and thank laboratory members in Kyoto University for their cooperation in the collection of plant material. This work was supported by Grants in Aid for Scientific Research (18201008) from the Japan Society for the Promotion of Science and the Global Environment Research Fund (F-094) of the Ministry of the Environment, Japan.

References

Ballou, F. & Lugon-Moulin, N. 2002. The estimation of population differentiation with microsatellite markers. Molec. Ecol. 11: 155–165.

Bowcock, A. M., A. Ruíz-Linares, J. Tomfohrde, E. Minch, J. R. Kidd & L. L. Cavalli-Sforza. 1994. High resolution of human evolutionary trees with polymorphic microsatellites. Nature 368: 455–457.

Cho, Y. G., T. Ishii, S. Temnykh, X. Chen, L. Lipovich, S. R. McCouch, W. D. Park, N. Ayres & S. Cartinhour. 2000. Diversity of microsatellites derived from genomic libraries and GenBank sequences in rice (*Oryza sativa* L.). Theor. Appl. Genet. 100: 713–722.

Dieringer, D. & C. Schlötterer 2003. Microsatellite analyser (MSA): a platform independent analysis tool for large microsatellite data sets. Molec. Ecol. Notes 3: 167–169.

Dolgin, E. S., B.Charlesworth, S. E. Baird & A. D. Cutter. 2007. Inbreeding and outbreeding depression in *Caenorhabditis nematodes*. Evolution 61: 1339–1352.

Ellstrand N. C. & D. R. Elam. 1993. Population genetic consequences of small population size: implications for plant conservation. Annual Rev. Ecol. Syst. 24: 214–242.

El Mousadik, A. & R. J. Petit. 1996. High level of genetic differentiation for allelic richness among populations of the argan tree [*Argania spinosa* (L.) Skeels] endemic to Morocco. Theor. Appl. Genet. 92: 832–839.

Frankham, R. & K. Ralls. 1998. Inbreeding leads to extinction. Nature 392: 441–442.

Fukushimagata Lagoon Water Park. 2007. International GORGON PLANT summit. p. 38. Fukushimagata Lagoon Water Park, Niigata (in Japanese).

Gao, H., S. Williamson & C. D. Bustamante. 2007. A Markov chain Monte Carlo approach for joint inference of population structure and inbreeding rates from multilocus genotype data. Genetics 176: 1635–1651.

Goldstein, D. B., A. R. Linares, L. L. Cavalli-Sforza & M. W. Feldman. 1995. Genetic absolute dating based on microsatellites and the origin of modern humans. Proc. Natl. Acad. Sci. USA 92: 6723–6727.

Goto, T. 2009. A new population of *Euryale ferox*. Kuroshio 27: 13 (in Japanese).

Goudet, J. 2001. FSTAT, a program to estimate and test gene diversities and fixation indices (version 2.9.3).

<http://www2.unil.ch/popgen/softwares/fstat.htm>.

Hada, Y. 1988. Regeneration of *Euryale ferox*. Bull. Water Plant Soc., Jpn 33/34: 31–33 (in Japanese).

Hufford, K. M. & S. J. Mazer. 2003. Plant ecotypes: genetic differentiation in the age of ecological restoration. Trends Ecol. Evol. 18: 147–155.

Hutchison, D. W. & A. R. Templeton. 1999. Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. Evolution 53: 1898–1914.

Imanishi, A., S. Kaneko, Y. Isagi, J. Imanishi, Y. Natuhara & Y. Morimoto. 2011. Development of microsatellite markers for *Euryale ferox* (Nymphaeaceae), an endangered aquatic plant species in Japan. Am. J. Bot. 98: e233–235.

Kadono, Y. 1994a. Aquatic plants of Japan. Bun-ichi Sogo Shuppan, Tokyo (in Japanese).

Kadono, Y. 1994b. Localities of *Euryale ferox* Salisb. Bull. Water Plant Soc., Jpn 53: 15–19 (in Japanese).

Kadono, Y. & E. L. Schneider. 1987. The life history of *Euryale ferox* Salisb. in south-western Japan with special reference to reproductive ecology. Plant Sp. Biol. 2: 109–115.

Kadono, Y. & M. Yuma. 1995. The nature of wetlands in Japan. pp. 136–137. Hoikusha, Osaka (in Japanese).

Koren, O. G., M. S. Yatsunskaya & O. V. Nakonechnaya. 2012. Low level of allozyme polymorphism in relict aquatic plants of the Far East *Nelumbo komarovii* Grossh. and *Euryale ferox* Salisb. Russ. J. Genet. 48: 912–919.

Matsumoto, S. 2002. Regeneration of *Euryale ferox* in 50 years. Bull. Water Plant Soc., Jpn 76: 35–39 (in Japanese).

Milligan, B. 1992. Plant DNA isolation. *In*: Hoelzel AR (ed.), Molecular genetic analysis of populations: a practical approach. pp. 59–88. IRL Press, Oxford.

Ministry of the Environment of Japan, 2012. Red List of Threatened Plants of Japan, <http://www.biodic.go.jp/english/rdb/rdb_f.html> [accessed 09 December 2013].

Newman, D. & D. Pilson. 1997. Increased probability of extinction due to decreased genetic effective population size: experimental populations of *Clarkia pulchella*. Evolution 51: 345–362.

Okada, Y. & T. Otaya. 1930. Study of *Euryale ferox* Salisb. VI. Cleistogamous versus chasmogamous flowers. Bot. Mag. (Tokyo) 44: 369–373.

Parker, M. A. 1992. Outbreeding depression in a selfing annual. Evolution 46: 837-841.

Peakall, R. & P. E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research—an update. Bioinformatics 28: 2537–2539.

Quan, Z., L. Pan, W. Ke & Y. Ding. 2009. Polymorphic microsatellite markers in *Euryale ferox* Salisb. (Nymphaeaceae). Molec. Ecol. Resour. 9: 330–332.

Schneider, E. L., S. C. Tucker & P. S. Williamson. 2003. Floral development in the Nymphaeales. Int. J. Plant Sci. 164: S279–S292.

Takagi, M. & I. Honda. 2012. Significance of the appearance of *Euryale ferox* in Noto Peninsula. Bull. Ishikawa Mus. Nat. Hist. 2: 57–58 (in Japanese).

Tanaka, N. 2012. Science of aquatic plants as heresy. pp. 230–241. Beret Publishing, Tokyo (in Japanese).

Uesugi, R., J. Nishihiro, Y. Tsumura & I. Washitani. 2007. Restoration of genetic diversity from soil seed banks in a threatened aquatic plant, *Nymphoides peltata*. Conserv. Genet. 8:111–121.

Received January 17, 2014; accepted October 7, 2014